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Molecular characterization of *Colletotrichum* species causing soybean anthracnose in Argentina

Araceli M. Ramos[1], Luis Franco Tadic[1], Isabel Cinto[1,*], Marcelo Carmona[2] & Marcela Gally[2]

[1] *Departamento BBE, Lab. 9, FCEN (UBA), PROPLAME-PRHIDEB (CONICET) &*
[2] *Cátedra de Fitopatología, Facultad de Agronomía, UBA C.A.B.A. Argentina*
* Correspondence to *isa.cinto@gmail.com* or *icinto@bg.fcen.uba.ar*

Abstract —Twenty-six isolates obtained from soybean crops (*Glycine max*) with typical anthracnose symptoms were identified as *Colletotrichum truncatum* (73 %) and *C. destructivum* (26 %). Their genetic relationships were studied using the AFLP method. A UPGMA phenogram divided the strains into two clusters corresponding with the two species. Genetic distances based on association coefficient were 0.71–0.89 among the 18 *C. truncatum* strains and 0.67–1 among the eight *C. destructivum* strains. Genetic variability within species, measured in terms of percentage of polymorphic loci, was high (<90%). Only two isolates showed 100% similarity, suggesting high intraspecific variability.

Key words —*Glomerella glycines*, teleomorph, genetic diversity

Introduction

Soybean (*Glycine max* (L.) Merr.) is the most important crop in Argentina, with late-season diseases caused by *Septoria glycines, Cercospora kikuchii, Phomopsis sojae, Colletotrichum destructivum,* and *C. truncatum* causing major losses (Carmona et al. 2009, 2011).

The two anthracnose pathogens most frequently associated with soybean are *Colletotrichum truncatum* (Schwein.) Andrus & W.D. Moore and *C. destructivum* O'Gara, [= *Glomerella glycines* Lehman & F.A. Wolf]. The traditional characterization and identification of *Colletotrichum* species has relied on morphological differences (Sutton 1992). Molecular markers constitute the current choice to obtain diagnostic characters as an aid for species-level identification and to evaluate genetic diversity. The amplified fragment length polymorphism (AFLP) method (Vos et al. 1995) is frequently used to monitor the identity of an isolate or to determine the degree of similarity among isolates, owing to certain advantages over other techniques (i.e. high level of identified polymorphism, high reproducibility, relative technical simplicity; Briad et

al. 2000). The taxonomy and nomenclature of the anthracnose pathogens (*Colletotrichum* spp.) is confusing (Hyde et al. 2009 a) and have recently been summarised by Hyde et al. (2009b), incorporating data obtained by DNA analysis and phylogenetic systematics.

Physiological studies have been performed on *C. truncatum* isolates obtained from soybean plants in Argentina (Levin et al. 2007, Ramos et al. 2010), but the genetic structure of anthracnose fungi associated with the Argentinean soybean crop is not available. Our study aimed to evaluate genetic variability and genetic relationships within *Colletotrichum* species obtained from symptomatic soybean plants in Argentina.

Materials & methods

Fungal Isolation

Diseased soybean plants showing typical anthracnose symptoms were collected from different geographic locations in Argentina. The isolates were obtained from lesions of stems and pods, using the procedure of Levin et al. (2007).

All fungal strains used in this survey were deposited at the Herbarium and Culture Collection of the Departamento de Biodiversidad, Facultad de Ciencias Exactas y Naturales Universidad de Buenos Aires (BAFC). Table 1 shows the collection locality and BAFC accession number of each strain used.

Table 1. *Colletotrichum truncatum* and *C. destructivum* fungal strains studied

Species	Collection locality*	Strains tested #
C. truncatum	Godeken, SF, Arg.	C1 (3093)
	Cerro Azul, SF, Arg.	C2 (3094)
	Armstrong, SF, Arg.	C4 (3096), C5 (3097)
	Cruz Alta, CO, Arg.	C6 (3098)
	Fac. Agronomía CABA, Arg.	C7 (3099)
	Lobos, BA, Arg.	C8 (3100), C9 (3101)
	Saenz Peña, CH, Arg.	C10 (3102)
	Salto, BA, Arg.	C14 (3396)
	Las Lajitas, SA, Arg.	C15 (3397)
	Piamonte, SF, Arg.	C26 (3398)
	Otamendi, BA, Arg.	C24 (3399), C32 (3448)
	Las Varillas, SF, Arg.	C25 (3400)
	Fac. Agronomía CABA, Arg.	C29 (3443), C30 (3444)
	Venado Tuerto, SF, Arg.	C31 (3447)
C. destructivum	Urdinarrain, ER, Arg.	C16 (3434)
	Chivilcoy, BA, Arg.	C17 (3435)
	Río Cuarto, CO, Arg.	C18 (3436)
	9 de Julio, BA, Arg.	C19 (3437)
	Barranca Yaco, SF, Arg.	C21 (3439)
	Piamonte, SF, Arg.	C23 (3441)
	Chivilcoy, BA. Arg.	C27 (3401), C28 (3442)

* Abbreviations: BA = Buenos Aires Province; CABA = Ciudad Autónoma de Buenos Aires; CH = Chaco Province; CO = Córdoba Province; ER = Entre Ríos Province; LR = La Rioja Province; SA = Salta Province; SF = Santa Fé Province; Arg.: = Argentina.

Numbers in parentheses correspond to the BAFC culture number (see Materials & methods).

Table 2. AFLP primers used in this study

Primers	Sequence 5´-3´
E+AC	GAC TGC GTA CCA ATT CAC
E+AA	GAC TGC GTA CCA ATT CAA
E+ACG	GAC TGC GTA CCA ATT CAC G
E+AAC	GAC TGC GTA CCA ATT CAA C
E+AAG	GAC TGC GTA CCA ATT CAA G
M+A	GAT GAG TCC TGA GTA AA
M+G	GAT GAG TCC TGA GTA AG
M+C	GAT GAG TCC TGA GTA AC
M+T	GAT GAG TCC TGA GTA AT

* E = *EcoRI*; M = *MseI*.

DNA extraction

Actively growing mycelia were obtained and then ground in liquid nitrogen (Ramos et al. 2000). Genomic DNA was extracted following Gottlieb & Lichtwardt (2001). Quality control and quantification of genomic DNA was carried out by agarose gel (0.8% w/v) electrophoresis and by comparison with a DNA molecular-size standard (Lambda EcoRI/HindIII, Promega Corp.). Gels, containing ethidium bromide, were photographed under UV light.

AFLP

The AFLP methodology was carried out on 250 ng of genomic DNA using the AFLP® Analysis System for Microorganisms Primer Kit (Invitrogen) as described in the instructions manual with minor modifications (Gottlieb et al. 2005). Selective primers used herein, containing one, two or three additional bases, were combined as in Table 2. All PCR amplifications were performed in a Techne Progene thermal cycler.

Polyacrylamide gel electrophoresis conditions were conducted as in Gottlieb et al. (2005). A 30-330-bp AFLP® DNA Ladder (Invitrogen) size marker was included twice in each electrophoresis run. Thus, the size of scored AFLP bands ranged from 90 to 330 bp. AFLP bands were visualized using the Silver Sequence ™ DNA Sequencing System (Promega). Air-dried gels were digitalized and visually analyzed using the Adobe Photoshop TM (Adobe Systems, Mountain View, CA, USA).

Data analysis

Each AFLP band, regardless of its relative intensity, was considered as a dominant allele at only one locus. The data, as either present (1) or absent (0), were extracted as a table. Monomorphic bands (bands present in all individuals of a species) were discriminated within each species and across the entire data set. The binary matrix was analyzed with the program NTSYS-PC version 2.02i (Rohlf 1993). The unweighted pair-group method with arithmetic mean (UPGMA) cluster analysis was performed based on the simple matching (SM), Jaccard (J), and Dice (D) association coefficients (Sneath & Sokal 1973). Phenograms showing similarity relations were generated by the same program, with the tree display option (TREE). Correlation cophenetic coefficients (r) were calculated for each association coefficient (Sokal & Rohlf 1962). A cophenetic correlation (r) of 0.8 was considered a good fit. A three-dimensional graphic was obtained with the Principal Coordinates ordination method (Gower 1966).

The percentage of polymorphic loci (P = number of polymorphic loci/ number of loci analyzed) for each primer combination was calculated.

Results

Twenty-six isolates obtained from soybean crops that showed typical anthracnose symptoms were identified as *Colletotrichum truncatum* (73%) and *C. destructivum* (26%), based on their morphological characteristics (Sutton 1992).

Characterization of AFLP markers

Six primers (Table 3) that produced high numbers of clear, reproducible bands by AFLP amplification were selected. The analysis of the six primers yielded a total of 812 bands that appeared consistently in all experiments among the amplified fragments of the 26 isolates. The size of the amplified bands ranged from 50 to 550 bp but we analyzed only bands from 90 to 330 bp.

The total number of AFLP bands (polymorphic and monomorphic) obtained and the percentage of polymorphic loci for each selective primer combination in two fungal species assayed are described in Table 3. All primers detected a high number of polymorphic bands within species and a high percentage of polymorphic loci was obtained.

Table 3. Total AFLP bands (polymorphic and monomorphic) detected for each primer combination in each assayed fungal species with polymorphic loci percentages (P%).

Primer combination	Species	Total N° of bands	Polymorphic bands	Monomorphic bands	P%
M+T/E+ACG	*C. truncatum*	73	65	8	89
	C. destructivum	81	74	7	91
M+C/E+AAG	*C. truncatum*	117	111	6	95
	C. destructivum	111	109	2	98
M+A/E+AAG	*C. truncatum*	95	93	2	98
	C. destructivum	56	51	5	91
M+A/E+ACG	*C. truncatum*	73	71	2	97
	C. destructivum	100	96	4	96
M+G/E+ACG	*C. truncatum*	82	80	2	97
	C. destructivum	54	52	2	96
M+A/E+AAC	*C. truncatum*	103	82	21	79
	C. destructivum	56	48	8	86

Only 41 (7.6%) of the bands obtained in *C. truncatum* and 28 (6.1%) in *C. destructivum* were monomorphic. Genetic variability within species, measured in terms of percentage of polymorphic loci, was high: *C. truncatum* (P = 92%) and *C. destructivum* (P = 94%).

The UPGMA phenogram (Figure 1) grouped all strains into two defined clusters with a SM coefficient of 0.6 between them. The main cluster included

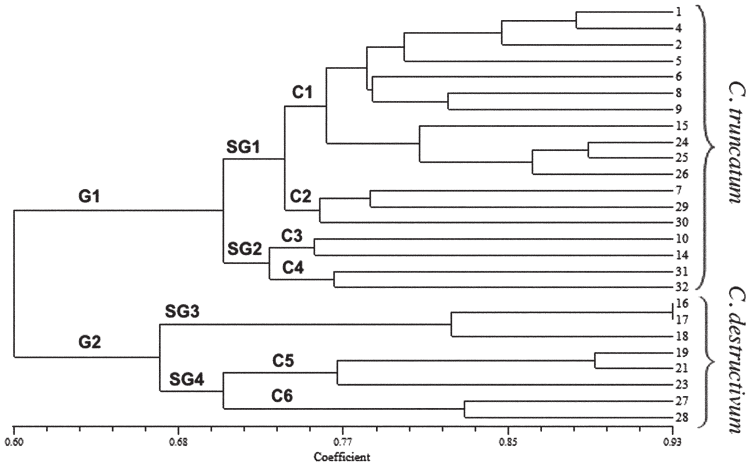

Figure 1. UPGMA phenogram showing relationships of *Colletotrichum truncatum* and *C. destructivum* isolates based on the simple matching (SM) association coefficient estimated from AFLP loci.

the 18 isolates identified as *C. truncatum* (Figure 1, G1), whereas the other (Figure 1, G2) included the eight isolates identified as *C. destructivum*. There was no direct correlation between molecular genotype and geographic origin.

Genetic distance and cluster analysis

The phenogram obtained using the UPGMA method was constructed based on Simple Matching (SM) coefficients. Little distortion occurred while constructing this phenogram, as implied by the value of the correlation cophenetic coefficient (r = 0.91903).

The main group (Figure 1, G1) comprised two subgroups (SG1 and SG2), one with 14 isolates and two conjuncts (C1 and C2), and the other (SG2) with two conjuncts (C3 and C4), each with two isolates. Group 2, formed by *C. destructivum* isolates, showed two subgroups, SG3 formed by three isolates (16, 17 and 18), and SG4, which included conjunct C5 and C6 formed by the rest of isolates belonging to *C. destructivum*. The relatedness of the 18 isolates of *C. truncatum* was 0.71–0.89, whereas that among the 8 isolates of *C. truncatum* was 0.67–1. Only two isolates (16 and 17, SG3) showed 100% similarity.

The three-dimensional graphic generated by the ordination technique distinguished two groups (Figure 2, GI and GII) and showed nearly the same relations between isolates as the phenogram (Figure 1). The first group included the 18 isolates belonging to *C. truncatum*, which are slightly differentiated in

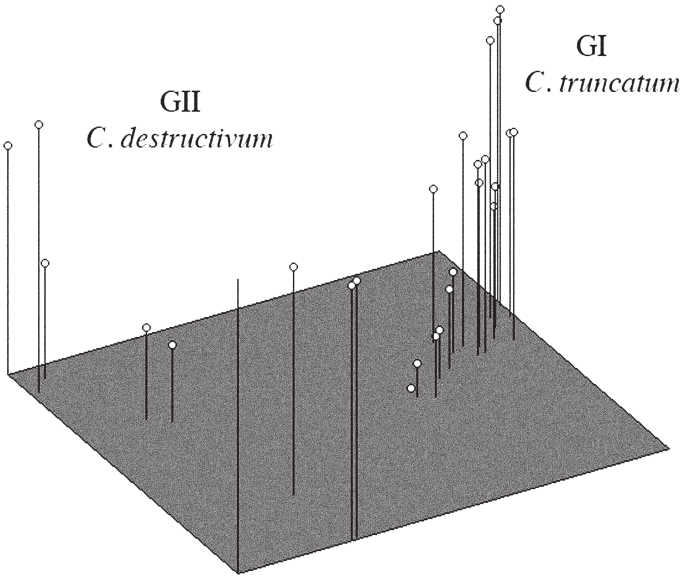

Figure 2. Three-dimensional graphic obtained with the Principal Coordinates ordination technique.

axis 1 and largely separated in the other two axes. The second group showed the seven isolates of *C. destructivum* largely separated in axis 1 and moderately differentiated in axes 2 and 3. Group GI included isolates of SGI and SGII of the phenogram and group GII comprised the isolates of SGIII and SGIV.

Discussion

C. truncatum and *C. destructivum* are highly variable pathogens in Argentina. Sharma (2009) also reported a high degree of variability among different isolates of *C. truncatum* studied through Random Amplified Polymorphic DNA (RAPD) analysis.

Perithecia of the *C. destructivum* teleomorph (the state referred to in dual nomenclature as *Glomerella glycines*) are frequently found in the fields of Argentina, indicating that the sexual mechanism is an important source of variability in the populations of this species. However, sexual recombination in most *Colletotrichum* species is rare in nature (Wharton & Diéguez-Uribeondo 2004; Hyde et al. 2009b).

Nevertheless, in our study, we found a high degree of variability in the populations analyzed. Alternatives to sexual recombination for genetic interaction among fungi are heterokaryosis and mitotic recombination (Esser

& Kuenen 1967). In many *Colletotrichum* populations, the only means of exchanging genetic material between two strains is mainly or exclusively vegetative, by means of anastomosis and heterokaryosis (Correll et al. 2000). These processes have been studied in some *Colletotrichum* species (Vaillancourt 1994; Correll et al. 1993; Elmer 2001; Nitzan et al. 2002; Rodriguez-Guerra et al. 2003; Barcelos et al. 2011). Sant'Anna et al. (2010) related genetic diversity analyzed by RAPD with the presence of vegetative compatibility groups in *C. truncatum*, and concluded that the parasexual process might be an important factor to increase the genetic variability in the pathogen.

Almost the entire soybean crop area of Argentina is cultivated under no-tillage systems. The crop debris remaining on the soil surface provides optimum conditions for the active development of reproductive structures of *C. truncatum* and *C. destructivum*. Any event of mutation or sexual or vegetative recombination is enhanced by the high reproductive rate occurring in these conditions. Gene flow and migration forces seem to be relatively unimportant due to the weak dispersal ability of the spores, as both conidia and ascospores are immersed in a mucous matrix, which prevents dispersal, by wind. The only possibility of migration to long distances is by means of seed transmission. Nevertheless, both species are not frequent seed pathogens in the Pampean Region.

The level of genetic variability within populations of plant pathogens provides an indication of their adaptability of pathogen in overcoming plant resistance and other measures used to control the pathogen, such as the application of fungicides in agro-ecosystems (McDonald et al. 1989; McDonald & Linde 2002). In our study, the high genetic diversity indicates increasing fitness of the population.

This paper constitutes the first report of genetic variability in *C. truncatum* and *C. destructivum* isolates collected from different soybean growing areas in Argentina.

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